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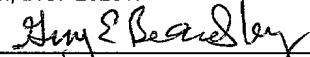
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APPLICATION
FOR
UNITED STATES LETTERS PATENT

APPLICANT : John Kisiday, Alan Grodzinsky and Shuguang Zhang
TITLE : PEPTIDE SCAFFOLD ENCAPSULATION OF TISSUE
CELLS AND USES THEREOF

PATENT
ATTORNEY DOCKET NO:01997/537001

PEPTIDE SCAFFOLD ENCAPSULATION OF TISSUE CELLS AND USES

5

THEREOF

Statement as to Federally Sponsored Research

This invention was made with government support under NIH grants AR45779 and AR33236. The government therefore has certain rights in the invention.

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Background of the Invention

During the past decade there has been substantial effort expended to

develop materials for the repair and replacement of various tissues, especially cartilage tissue in the knee joint. Although various polymeric biomaterials have been developed for tissue repair, these biomaterials suffer from immune

15 incompatibility and improper distribution of stress. Furthermore, the use of material from animals, such as cow hide or cartilage from pigs or sharks, has raised concerns of possible contamination by infectious agents, such as prions.

Thus, improved materials of biological origin that have improved compatibility, present a reduced risk of contamination, and provide the proper biomechanical

20 characteristics for tissue repair are needed. In addition, these materials will preferably promote the interaction between native tissue and implanted cells. The ability to control the rate of biodegradation of these material is also desirable.

Summary of the Invention

The purpose of the present invention is to provide improved biomaterials

25 and methods for tissue repair or replacement. We discovered that living cells may

be encapsulated by a biodegradable peptide scaffold in a three-dimensional arrangement of predetermined geometry. The secretion of extracellular matrix components by the encapsulated cells significantly increases the stiffness of the scaffolds and thereby improves the ability of the scaffold to repair or replace 5 endogenous cartilage.

Accordingly, in one aspect, the invention features a macroscopic scaffold having amphiphilic peptides. The peptides have alternating hydrophobic and hydrophilic amino acids, are complementary and structurally compatible, and self-assemble into a beta-sheet macroscopic scaffold. The macroscopic scaffold 10 encapsulates living cells, and the encapsulated cells are present in the macroscopic scaffold in a three-dimensional arrangement.

In another aspect, the invention features a method of forming a macroscopic scaffold. This method involves incubating peptides and living cells in an aqueous solution having an iso-osmotic solute, preferably under conditions 15 that do not allow the peptides to substantially self-assemble. Preferably, the solution contains less than 10, 5, 1, or 0.1 mM electrolyte or is substantially free of electrolyte. The peptides have alternating hydrophobic and hydrophilic amino acids and are complementary and structurally compatible. Sufficient electrolyte is added to the solution to initiate self-assembly of the peptides into a beta-sheet 20 macroscopic scaffold, whereby the cells are encapsulated by the formation of the macroscopic scaffold. The encapsulated cells are present in the macroscopic scaffold in a three-dimensional arrangement. Preferably, the concentration of the added electrolyte is at least 5, 10, 20, or 50 mM. Preferred electrolytes include 25 Li^+ , Na^+ , K^+ , and Cs^+ . In one preferred embodiment, the concentration of the iso-osmotic solute is at least 50, 150, or 300 mM. In another preferred embodiment,

the concentration of the iso-osmotic solute is contained in one of the following ranges 200 to 250 mM, 250 to 270 mM, 270 to 300 mM, 300 to 400 mM, 400 to 500 mM, 500 to 600 mM, 600 to 700 mM, 700 to 800 mM, or 800 to 900 mM, inclusive. Preferred iso-osmotic solutes include carbohydrates, such as

5 monosaccharides or disaccharides. Examples of preferred carbohydrates include sucrose, glucose, galactose, fructose, ribose, mannose, arabinose, and xylose. Still another preferred iso-osmotic solute is glycerol, such an aqueous solution of glycerol that is between 5 to 20% (v/v) glycerol.

In yet another aspect, the invention provides a method of forming a

10 macroscopic scaffold of predetermined shape or volume. This method includes incubating peptides and living cells in an aqueous solution having an iso-osmotic solute, preferably under conditions that do not allow the peptides to substantially self-assemble. Preferably, the solution contains less than 10, 5, 1, or 0.1 mM electrolyte or is substantially free of electrolytes. The solution is contained in a
15 pre-shaped mold dimensioned to determine the volume or shape of the macroscopic scaffold. The peptides have alternating hydrophobic and hydrophilic amino acids and are complementary and structurally compatible. Sufficient electrolyte is added to the solution to initiate self-assembly of the peptides into a beta-sheet macroscopic scaffold, whereby the cells are encapsulated by the
20 formation of the macroscopic scaffold. The encapsulated cells are present in the macroscopic scaffold in a three-dimensional arrangement. Preferably, the concentration of the added electrolyte is at least 5, 10, 20, or 50 mM. Preferred electrolytes include Li^+ , Na^+ , K^+ , and Cs^+ . In one preferred embodiment, the concentration of the iso-osmotic solute is at least 50, 150, or 300 mM. In another
25 preferred embodiment, the concentration of the iso-osmotic solute is contained in

one of the following ranges 200 to 250 mM, 250 to 270 mM, 270 to 300 mM, 300 to 400 mM, 400 to 500 mM, 500 to 600 mM, 600 to 700 mM, 700 to 800 mM, or 800 to 900 mM, inclusive. Preferred iso-osmotic solutes include carbohydrates, such as monosaccharides or disaccharides. Examples of preferred carbohydrates

5 include sucrose, glucose, galactose, fructose, ribose, mannose, arabinose, and xylose. Still another preferred iso-osmotic solute is glycerol, such an aqueous solution of glycerol that is between 5 to 20% (v/v) glycerol.

In still another aspect, the invention features a method of regenerating a tissue. This method includes administering to a mammal a macroscopic scaffold

10 having amphiphilic peptides and encapsulated living cells. The peptides have alternating hydrophobic and hydrophilic amino acids, are complementary and structurally compatible, and self-assemble into a beta-sheet macroscopic scaffold.

The encapsulated cells are present in the macroscopic scaffold in a three-

dimensional arrangement. Preferably, the method is used to treat or prevent a

15 cartilage defect, connective tissue defect, nervous tissue defect, epidermal lining defect, endothelial lining defect, or arthritis. A preferred connective tissue is a ligament or tendon, and a preferred epidermal lining is skin. Preferably, the cells are autologous or allogeneic. Preferred routes of administration include oral, percutaneous, intramuscular, intravenous, subcutaneous, and surgical. A preferred

20 surgical administration is arthroscopic surgery. Preferably, the mammal is human.

In a related aspect, the invention provides another method of regenerating a tissue. This method involves administering to a mammal a solution having

amphiphilic peptides, living cells, and an iso-osmotic solute. The peptides have

alternating hydrophobic and hydrophilic amino acids and are complementary and

25 structurally compatible. The peptides do not substantially self-assemble prior to

administration, but they self-assemble into a beta-sheet macroscopic scaffold after administration to the mammal. The formation of the macroscopic scaffold encapsulates the cells *in vivo*, and the encapsulated cells are present in the macroscopic scaffold in a three-dimensional arrangement. Preferably, the

5 administered solution contains less than 10, 5, 1.0, or 0.1 mM electrolyte or is substantially free of electrolyte. Preferably, the concentration of the iso-osmotic solute is at least 50, 150, or 300 mM. In another preferred embodiment, the concentration of iso-osmotic solute is contained in one of the following ranges 200 to 250 mM, 250 to 270 mM, 270 to 300 mM, 300 to 400 mM, 400 to 500
10 mM, 500 to 600 mM, 600 to 700 mM, 700 to 800 mM, or 800 to 900 mM, inclusive. Preferred iso-osmotic solutes include carbohydrates, such as monosaccharides or disaccharides. Examples of preferred carbohydrates include sucrose, glucose, galactose, fructose, ribose, mannose, arabinose, and xylose. Still another preferred iso-osmotic solute is glycerol, such an aqueous solution of
15 glycerol that is between 5 to 20% (v/v) glycerol. Preferably, this method is used to treat or prevent a cartilage defect, connective tissue defect, nervous tissue defect, epidermal lining defect, endothelial lining defect, or arthritis. A preferred connective tissue is a ligament or tendon, and a preferred epidermal lining is skin. Preferably, the cells are autologous or allogeneic. Preferred routes of
20 administration include oral, percutaneous, intramuscular, intravenous, subcutaneous, and surgical. A preferred surgical administration is arthroscopic surgery. Preferably, the mammal is human.

In preferred embodiments of various aspects of the invention, the macroscopic scaffold is enzymatically degradable. In other preferred embodiments, the macroscopic scaffold is cleaved by a metalloprotease,
25

collagenase, or aggrecanase *in vivo* or *in vitro*. Preferably, an enzyme capable of cleaving the scaffold is produced by the encapsulating cells or nearby cells. In yet other preferred embodiments, the macroscopic scaffold further encapsulates a therapeutically active compound or chemoattractant. Examples of such

5 therapeutically active compounds include synthetic organic molecules, naturally occurring organic molecules, nucleic acid molecules, biosynthetic proteins, or modified naturally occurring proteins. In still other preferred embodiments, the macroscopic scaffold further encapsulates a growth factor, such as a cartilage-derived growth factor, transforming growth factor- β , platelet derived growth factor, insulin-like growth factor, acidic fibroblast growth factor, basic fibroblast growth factor, epidermal growth factor, hepatocytic growth factor, keratinocyte growth factor, or bone morphogenic protein. Preferred macroscopic scaffolds have peptides which include an adhesion site, growth factor binding site, growth factor, or sequence that provides targeting to a cell, tissue, organ, organ system, or site within an mammal. Other preferred macroscopic scaffolds have a pre-determined volume or shape. In preferred embodiments, the encapsulated cells are substantially uniformly distributed. In other preferred embodiments, the encapsulated cells are neurons, and the macroscopic scaffold allows axonal outgrowth by the neurons. Preferably, the axons extend beyond the surface of the macroscopic scaffold. In yet other preferred embodiments, axons from neurons outside of the macroscopic scaffold extend into the macroscopic scaffold. In still other preferred embodiments, the cells differentiate after encapsulation by the macroscopic scaffold. Preferably, cells such as bone marrow cells, perosteal cells, perichondrial cells, or embryonic stem cells differentiate into cartilage cells. In other preferred embodiments, the cells are polypotent or pluripotent. In still yet

other preferred embodiments, the cells are differentiated prior to encapsulation by the macroscopic scaffold and remain differentiated after the encapsulation.

Preferred cells include chondrocytes, bone marrow cells, perosteal cells, perichondrial cells, fibroblasts, neuronal cells, hippocampal cells, epidermal cells,

5 endothelial cells, keratinocytes, basal cells, spinous cells, granular cells, embryonic stem cells, ovarian cells, pancreatic cells, cervical cells, liver cells, and foreskin cells. The cells may be from any suitable source such as a human or bovine cells. Source of the cells may also include fetal or adult mammals or established cell lines.

10 Preferably, at least 40, 50, 60, 70, 80, 90, or 95% of the encapsulated cells are viable 1, 2, 4, 6, or more weeks after formation of the macroscopic scaffold.

In another preferred embodiment, at least 80 or 90% of the encapsulated cells are viable one day or one week after formation of the macroscopic scaffold. More preferably, at least 90% or 95% of the encapsulated cells are viable 6 weeks after

15 formation of the macroscopic scaffold. Preferably, the number of living cells encapsulated by the macroscopic scaffold five days after formation of the macroscopic scaffold is at least 1, 1.5, 3, 5, or 10 million per ml of the volume of the macroscopic scaffold. In another preferred embodiment, the number of living cells encapsulated by the macroscopic scaffold 3, 5, 10, 15, or more days after

20 scaffold formation is at least 2, 3, 5, 10, or 20-fold greater than the initial number of encapsulated cells. In yet another preferred embodiment, at least 60, 70, 80, 90, or 95% of the encapsulated cells are in cell-cell contact with another encapsulated cell or with a cell outside of the scaffold.

In other preferred embodiments, the encapsulated cells secrete extracellular

25 matrix components. Preferably, the secretion of extracellular matrix components

increases the equilibrium compression modulus of the macroscopic scaffold by at least 5, 10, 20, 30, 50, 100, 200, 300, 400, or 500 kPa or by at least 2, 5, 25, 50, 75, or 100-fold. Preferably, the extracellular matrix secreted by a cell is in contact with the extracellular matrix secreted by another cell. More preferably, the

5 extracellular matrix secreted by at least 60, 70, 80, 90, or 95% of the encapsulated cells is in contact with the extracellular matrix secreted by another encapsulated cell or secreted by a cell outside of the scaffold. Preferred macroscopic scaffolds further include a biodegradable sealant, glue, or polymer attached to the surface of the macroscopic scaffold that increases the equilibrium compression modulus of

10 the macroscopic scaffold.

In preferred methods of the invention, the macroscopic scaffold is subjected to a predetermined compression scheme. For example, the macroscopic scaffold may be subjected to constant or variable pressure for a predetermined amount of time, such as for as few as 1, 2, or 3 weeks to as long as 6, 8, or more weeks. A preferred compression scheme includes dynamic compression at 0.01 to 3 Hz or more preferably 0.1 to 1 Hz, superimposed on a static offset compression. Typically, the dynamic strain amplitude is between 0.01 and 10%, preferably, between 1 and 5%, and, more preferably, between 3 and 5%, and the static offset compression is between 5 and 15%. Preferably, the compression scheme

15 increases the equilibrium compression modulus of the macroscopic scaffold by at least 5, 10, 20, 30, 50, 100, 200, 300, 400, or 500 kPa or by at least 2, 5, 25, 50, 75, or 100-fold compared to a control macroscopic scaffold not subjected to the compression scheme. In another preferred embodiment, the compression scheme induces the secretion of extracellular matrix components by the cells.

20

25 Preferred peptides forming the macroscopic scaffold contain between 8 and

200 amino acids, 8 to 36 amino acids, or 8 to 16 amino acids, inclusive.

Preferably, the concentration of the peptides is between 1 and 10 mg/ml or between 4 and 8 mg/ml, inclusive. Preferably, the macroscopic scaffold is pre-shaped to interfit a tissue defect. In one preferred embodiment, the tissue defect is

5 in a joint, such as a knee, hip, shoulder, wrist, finger, ankle, toe, elbow, or neck.

Preferably the tissue is an epithelial, connective, muscular, or nervous tissue. In one preferred embodiment, the cartilage is articular, costal, fibrous, hyaline, semilunar, thyroid, or elastic cartilage. In another preferred embodiment, the scaffold does not elicit an adverse immune or inflammatory response.

10 It is also contemplated that the methods of the present invention may be used to repair an injury to an organ or muscle or to form an organ or muscle. Preferred organs include the bladder, brain, esophagus, fallopian tube, heart, intestines, gallbladder, kidney, liver, lung, ovaries, pancreas, prostate, spinal cord, spleen, stomach, testes, thymus, thyroid, trachea, ureter, urethra, and uterus.

15 By “scaffold” is meant a three-dimensional structure capable of encapsulating cells. The beta-sheet secondary structure of the scaffold may be confirmed using standard circular dichroism to detect an absorbance minimum at approximately 218 nm and a maximum at approximately 195 nm. Preferably, the scaffold is formed from the self-assembly of peptides that include L-amino acids,

20 D-amino acids, natural amino acids, non-natural amino acids, or a combination thereof. If L-amino acids are present in the scaffold, degradation of the scaffold produces amino acids which may be reused by the host tissue. It is also contemplated that the peptides may be covalently linked to a compound, such as a chemoattractant or a therapeutically active compound. The peptides may be 25 chemically synthesized or purified from natural or recombinant sources, and the

amino- and carboxy-termini of the peptides may be protected or not protected. The peptide scaffold may be formed from one or more distinct molecular species of peptides which are complementary and structurally compatible with each other. Peptides containing mismatched pairs, such as the repulsive pairing of two

5 similarly charged residues from adjacent peptides, may also form scaffolds if the disruptive force is dominated by stabilizing interactions between the peptides.

By "complementary" is meant the capable of forming ionic or hydrogen-bonding interactions between hydrophilic residues from adjacent peptides in the scaffold, as illustrated in Fig. 1. Preferably, each hydrophilic residue in a peptide

10 either hydrogen-bonds or ionically pairs with a hydrophilic residue on an adjacent peptide or is exposed to solvent.

By "structurally compatible" is meant capable of maintaining a sufficiently constant interpeptide distance to allow scaffold formation. Preferably the variation in the interpeptide distance is less than 4, 3, 2, or 1 Å. It is also contemplated that larger variations in the interpeptide distance may not prevent scaffold formation if sufficient stabilizing forces are present. This distance may be calculated based on molecular modeling or based on a simplified procedure that has been previously reported (U.S. Patent Number 5,670,483). In this method, the interpeptide distance is calculated by taking the sum of the number of unbranched atoms on the side-chains of each amino acid in a pair. For example, the interpeptide distance for a lysine-glutamic acid ionic pair is 5+4=9 atoms, and the distance for a glutamine-glutamine hydrogen-bonding pair is 4+4=8 atoms.

15 Using a conversion factor of 3 Å per atom, the variation in the interpeptide distance of peptides having lysine-glutaminic acid pairs and glutamine-glutamine

20 pairs (i.e., 9 versus 8 atoms) is 3 Å.

25

By “three-dimensional arrangement” is meant existing in three dimensions.

Cells having a three-dimensional arrangement are not all part of the same monolayer. As used herein, a monolayer is a cross section through the peptide scaffold that has a thickness equal to the average diameter of the encapsulated

5 cells and that includes at least one encapsulated cell. In one preferred embodiment, the encapsulated cells are neurons, and the average diameter of the neurons is determined by measuring the average diameter of the cell bodies of the neurons. An encapsulated cell is considered part of the monolayer if at least 51% of the volume of the cell is contained in the monolayer. Preferably, immediately 10 after scaffold formation, at least one monolayer contains less than 75, 50, 25, 20, 15, 10, 5, or 1% (in order of preference) of the encapsulated cells. More preferably, immediately after scaffold formation, less than 75, 50, 25, 20, 15, 10, 5, or 1% (in order of preference) of the encapsulated cells are part of the same monolayer.

15 By “substantially uniformly distributed” is meant that immediately after scaffold formation the center of mass of at least 50, 60, 70, 80, 90, or 100% of the cells encapsulated by the scaffold are separated from each other by distances that vary by less than 500, 100, 50, 20, or 10 μM . Preferably, 1, 2, 3, or 4 weeks after scaffold formation, the center of mass of cell clusters or cell pairs for at least 50, 20 60, 70, 80, 90, or 100% of the encapsulated cells are separated from each other by distances that vary by less than 500, 100, 50, 20, or 10 μM .

By “iso-osmotic solute” is meant a non-ionizing compound dissolved in an aqueous solution.

By “solution that is substantially free of electrolytes” is meant a solution in

25 which no electrolytes have been added or in which the concentration of

electrolytes is less than 0.01 or 0.001 mM.

The present invention provides a number of advantages related to the repair or replacement of tissues. For example, these methods enable the encapsulation of living cells by a peptide scaffold in a three-dimensional arrangement and in a substantially uniform distribution, which may promote the viability and proliferation of the cells. The peptide scaffolds also have the advantage of not eliciting a detectable immune or inflammatory response in mammals. Further, the peptide scaffolds exhibited no detectable swelling when the scaffold was added to a saline solution. This lack of swelling is probably due to the high water content of the scaffold (>99%). This unique property of the scaffold reduces the probability of an unregulated expansion of the scaffold that could lead to adverse physiological effects on neighboring tissues. Moreover, if desired, the *in vivo* rate of degradation of the scaffolds may be modulated by the incorporation of protease cleavage sites into the scaffold. The secretion of extracellular matrix components by the encapsulated chondrocytes increased the stiffness of the scaffold by over 50-fold, improving the ability of the scaffold to be used to replace endogenous cartilage. Furthermore, adding growth factors to the scaffold to stimulate the chondrocytes, compressing the scaffold, or increasing the initial concentrations of the cells or peptides may further increase the stiffness of the scaffold.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

Fig. 1 is a schematic illustration of the interactions between peptides in the peptide scaffold. Various peptides with amino acid sequences of alternating

hydrophobic and hydrophilic residues self-assemble to form a stable scaffold of beta-sheets when exposed to physiologically-equivalent electrolyte solutions (U.S. Patent Numbers 5,955,343 and 5,670,483). The peptide scaffolds are stabilized by numerous interactions between the peptides. For example, the positively 5 charged and negatively charged amino acid side chains from adjacent peptides form complementary ionic pairs, and other hydrophilic residues such as asparagine and glutamine participate in hydrogen-bonding interactions. The hydrophobic groups on adjacent peptides participate in van der Waals interactions. The amino and carbonyl groups on the peptide backbone also participate in intermolecular 10 hydrogen-bonding interactions.

Fig. 2 is a bar graph showing the rates of protein and proteoglycan synthesis in chondrocytes encapsulated by a peptide scaffold compared to the corresponding rates in chondrocytes suspended in an agarose gel.

Fig. 3 is a graph showing the amount of glycosaminoglycan (GAG) synthesized by chondrocytes encapsulated by a peptide scaffold or suspended in an agarose gel.

Fig. 4 is a picture of the toluidine blue staining of glycosaminoglycan in a peptide scaffold encapsulating chondrocytes.

Fig. 5 is a picture of immunohistochemical staining for collagen II in a peptide scaffold encapsulating chondrocytes.

Fig. 6 is a schematic illustration of the apparatus used for mechanical testing of peptide scaffolds and that may be used, if desired, to compress the scaffolds to stimulate the secretion of extracellular matrix components by the encapsulated cells (Buschmann *et al.*, J. of Cell Science 108:1497-1508, 1995)

Fig. 7 is a graph of the equilibrium stress values at different compressive

strain values for the peptide scaffold encapsulating chondrocytes. Based on this graph, a equilibrium modulus of 27 kPa was calculated, which is significantly higher than the equilibrium modulus of approximately 0.5 kPa in the absence of cells.

5 Fig. 8 is a picture showing the distribution of chondrocytes five days after scaffold formation. Each cluster or pair of cells evolved from one cell and is substantially uniformly dispersed.

10 Figs. 9A-9C are pictures of peptide scaffolds formed in a variety of predetermined shapes, including a tape (Fig. 9A), a rope (Fig. 9B), and a sheet (Figs. 9C) (Holmes *et al.*, PNAS 97:6728-6733, 2000). To form a tape-shaped macroscopic peptide scaffold, the RAD16-II peptide was dissolved in water and injected through a device, which consisted of two pieces of thin wire spaced 5 mm apart and sandwiched between two glass slides, into phosphate-buffered saline (PBS). The scaffold was then stained with Congo red. The tape shown in Fig. 9A is approximately 8 cm in length, 0.5 cm in width, and 0.3 mm in thickness. A similar procedure was also used to form a peptide scaffold in the shape of a rope. In this case, the aqueous peptide solution was introduced into PBS using a 3-mL syringe. The peptide scaffold rope shown in Fig. 9B is 18 cm in length and 2 mm in diameter. As illustrated in Fig. 9C, a peptide scaffold was also fabricated in the 15 shape of a sheet. A centimeter scale is shown below the sheet structure in Fig. 9C.

20

Detailed Description

To maintain their phenotype, chondrocytes are typically cultured in a three-dimensional environment. Within such an arrangement, chondrocytes develop a mechanically functional extracellular matrix and respond appropriately

to static and dynamic compressive loads. We have discovered that a peptide scaffold that encapsulates living cells in a three-dimensional arrangement may be formed by first mixing the cells and the peptides in a solution having the required osmolarity to maintain cell viability, and then adding sufficient electrolytes to

5 initiate self-assembly of the scaffold. Long-term cultures showed that the chondrocytes encapsulated by this scaffold deposited a continuous matrix, maintained a rounded morphology, and had a significant rate of protein and proteoglycan synthesis. These results indicate that a peptide gel scaffold encapsulating chondrocytes may be used to repair or replace cartilage tissue.

10 Secretion of extracellular matrix components by the encapsulated chondrocytes increased the equilibrium modulus, a measure of the strength of the scaffold, by over 50-fold by day 28 after scaffold formation. If desired, the stiffness of the scaffold may be further increased by incorporating cysteines which may be disulfide bonded or by incorporating aromatic residues which may be UV

15 cross-linked into the scaffold. In addition, varying the length or concentration of the peptides may further increase the stiffness of the scaffold. Moreover, forming the scaffolds in the presence of growth factors so that they are encapsulated by the scaffold, adding growth factors to the media surrounding the scaffold so that they diffuse into the scaffold, or using standard molecular biology techniques to

20 modify the encapsulated cells so that they express heterologous growth factors or over-express endogenous growth factors is expected to promote the proliferation of the encapsulated cells and to increase the secretion of extracellular matrix components by the cells. Moreover, subjecting the scaffold to external pressure may further enhance the secretion of extracellular matrix components by the cells,

25 resulting in an even higher equilibrium modulus. Also, the stiffness of the

scaffold may further increase after it is implanted *in vivo*. Thus, the equilibrium compression modulus of the peptide scaffold may approach the 500 kPa value of articular cartilage.

Because these peptide scaffolds have been previously shown to be

5 nontoxic to a variety of mammalian cell types, the methods of the present invention may also be applied to other cell types for applications involving other tissue types (Zhang *et al.*, *Biomaterials* 16:1385-1393, 1995). The strength of scaffolds that is required to repair or replace soft tissues such as young male thigh and forearm skin which have equilibrium compression moduli of 1.99 and 1.51
10 kPa, respectively, is much lower than that required for cartilage. Additionally, neurons grown in a monolayer on the outside surface of a peptide scaffold have been previously shown to exhibited extensive neurite outgrowth. Thus, neurons that are encapsulated by these peptide scaffolds using the methods of the present invention may project axons that enable cell-to-cell contact between the
15 encapsulated cells and neighboring endogenous neurons.

The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

Peptide Scaffolds

Certain peptides consisting of alternating hydrophilic and hydrophobic
20 amino acids self-assemble to form an exceedingly stable beta-sheet macroscopic scaffold in the presence of electrolytes, such as monovalent alkaline cations (U.S. Patent Numbers 5,955,343 and 5,670,483). For example, NaCl at a concentration of between 5 mM and 5 M induces the assembly of scaffolds within a few minutes. Lower concentrations of NaCl may also induce assembly but at a slower

rate. The side-chains of the peptides in the scaffold partition into two faces, a polar face with charged ionic side chains and a nonpolar face with alanines or other hydrophobic groups. These ionic side chains are self-complementary to one another in that the positively charged and negatively charged amino acid residues 5 can form complementary ionic pairs. These peptides are therefore called ionic self-complementary peptides, or Type I self-assembling peptides. If the ionic residues alternate with one positively and one negatively charged residue (- + - + - + - +), the peptides are described as “modulus I;” if the ionic residues alternate with two positively and two negatively charged residues (- - + + - - + +), the 10 peptides are described as “modulus II.”

Many modulus I and II self-complementary peptides with identical compositions and length; such as EAK16, KAE16, RAD16, RAE16, and KAD16; have been analyzed previously (Table 1). Modulus IV ionic self-complementary peptides containing 16 amino acids; such as EAK16-IV, KAE16-IV, DAR16-IV 15 and RAD16-IV; have also been studied. If the charged residues in these self-assembling peptides are substituted (*i. e.*, the positive charged lysines are replaced by positively charged arginines and the negatively charged glutamates are replaced by negatively charged aspartates), there are essentially no significant effects on the self-assembly process. However, if the positively charged residues, 20 lysine and arganine are replaced by negatively charged residues, aspartate and glutamate, the peptides can no longer undergo self-assembly to form macroscopic scaffolds; however, they can still form a beta-sheet structure in the presence of salt. Other hydrophilic residues, such as asparagine and glutamine, that form hydrogen-bonds may be incorporated into the peptides instead of, or in addition 25 to, charged residues. If the alanines in the peptides are changed to more

hydrophobic residues, such as leucine, isoleucine, phenylalanine or tyrosine, these peptides have a greater tendency to self-assemble and form peptide matrices with enhanced strength. Some peptides that have similar compositions and lengths as the aforementioned peptides form alpha-helices and random-coils rather than 5 beta-sheets and do not form macroscopic structures. Thus, in addition to self-complementarity, other factors are likely to be important for the formation of macroscopic scaffolds, such as the peptide length, the degree of intermolecular interaction, and the ability to form staggered arrays.

Table 1. Representative Self-Assembling Peptides

	Name	Sequence (n-->c)	Modulus	Structure
5	RADA16-I	n-RADARADARADARADA-c	I	β
	RGDA16-I	n-RADARGDARADARGDA-c	I	r.c.
	RADA8-I	n-RADARADA-c	I	r.c.
	RAD16-II	n-RARADADARARADADA-c	II	β
	RAD8-II	n-RARADADA-c	II	r.c.
10	EAKA16-I	n-AEAKAEAKAEAKAEAK-c	I	β
	EAKA8-I	n-AEAKAEAK-c	I	r.c.
	RAEA16-I	n-RAEAREAREAREAEA-c	I	β
	RAEA8-I	n-RAEAREEA-c	I	r.c.
	KADA16-I	n-KADAKADAKADAKADA-c	I	β
15	KADA8-I	n-KADAKADA-c	I	r.c.
	EAH16-II	n-AEAEAHAHAEAEAHAH-c	II	β
	EAH8-II	n-AEAEAHAH-c	II	r.c.
	EFK16-II	n-FEFEFKFKFEFEFKFK-c	II	β
	EFK8-II	n-FEFKFEFK-c	I	β
20	ELK16-II	n-LELELKLIKLELELKLIK-c	II	β
	ELK8-II	n-LELELKLIK-c	II	r.c.
	EAK16-II	n-AEAEAKAKAEAEAKAK-c	II	β
	EAK12	n-AEAEAEAEAKAK-c	IV/II	α/β
	EAK8-II	n-AEAEAKAK-c	II	r.c.
25	KAE16-IV	n-KAKAKAKAEAEAEAEA-c	IV	β

EAK16-IV	n-AEAEAEAEAKAKAKAK-c	IV	β	
RAD16-IV	n-RARARARADADADADA-c	IV	β	
DAR16-IV	n-ADADADADARARARAR-c	IV	α/β	
DAR16-IV*	n-DADADADARARARARA-c	IV	α/β	
5	DAR32-IV	n-(ADADADADARARARAR)-c	IV	α/β
	EHK16	n-HEHEHKHKHEHEHKHK-c	N/A	r.c.
	EHK8-I	n-HEHEHKHK-c	N/A	r.c.
	VE20*	n-VEVEVEVEVEVEVEVE-c	N/A	β
	RF20*	n-RFRFRFRFRFRFRFRFRF-c	N/A	β

10

“β” denotes beta-sheet; “α” denote alpha-helix; “r.c.” denotes random coil; “N/A” denotes not applicable. *Both VE20 and RF20 form a beta-sheet when they are incubated in a solution containing NaCl; however, they do not self-assemble to form macroscopic scaffolds.

15 Other self-assembling peptides may be generated by changing the amino acid sequence of any self-assembling peptide by a single amino acid residue or by multiple amino acid residues. Additionally, the incorporation of specific cell recognition ligands, such as RGD or RAD, into the peptide scaffold may promote the proliferation of the encapsulated cells. *In vivo* these ligands may also attract
 20 cells from outside a scaffold to the scaffold, where they may invade the scaffold or otherwise interact with the encapsulated cells. To increase the mechanical strength of the scaffolds, cysteines may be incorporated into the peptides to allow the formation of disulfide bonds, or residues with aromatic rings may be incorporated and cross-linked by exposure to UV light. The *in vivo* half-life of
 25 the scaffolds may also be modulated by the incorporation of protease cleavage

sites into the scaffold, allowing the scaffold to be enzymatically degraded.

Combinations of any of the above alterations may also be made to the same peptide scaffold.

Peptides capable of being cross-linked may be synthesized using standard

5 f-moc chemistry and purified using high pressure liquid chromatography (Table 2). The formation of a peptide scaffold may be initiated by the addition of electrolytes as described herein. The hydrophobic residues with aromatic side chains may be crossed linked by exposure to UV irradiation. The extent of the cross-linking may be precisely controlled by the predetermined length of exposure
10 to UV light and the predetermined peptide concentration. The extent of cross-linking may be determined by light scattering, gel filtration, or scanning electron microscopy using standard methods. Furthermore, the extent of cross-linking may also be examined by HPLC or mass spectrometry analysis of the scaffold after digestion with a protease, such as matrix metalloproteases. The
15 material strength of the scaffold may be determined before and after cross-linking, as described herein.

Table 2. Representative Peptides for Cross-Linking Study

<u>Name</u>	<u>Sequence (N-->C)</u>
RGDY16	RGDYRYDYRYDYRGDY
20 RGDF16	RGDFRFDFRFDFRGDF
RGDW16	RGDWRWDWRWDWRGDW
RADY16	RADYRYEYRYEYRADY
RADF16	RADFDFDFRADF
RADW16	RADWRWDWRWDWRADW

Aggrecan processing sites, such as those underline in Table 3, may be added to the amino- or carboxy-terminus of the peptides or between the amino- and carboxy-termini. Likewise, other matrix metalloproteases (MMPs) cleavage sites, such as those for collagenases, may be introduced in the same manner.

5 Peptide scaffolds formed from these peptides, alone or in combination with peptides capable of being cross-linked, may be exposed to various protease for various lengths of time and at various protease and scaffold concentrations. The rate of degradation of the scaffolds may be determined by HPLC, mass spectrometry, or NMR analysis of the digested peptides released into the

10 supernatant at various time points. Alternatively, if radiolabeled peptides are used for scaffold formation, the amount of radiolabeled peptides released into the supernatant may be measured by scintillation counting.

Table 3. Representative Peptides for Enzymatic Cleavage Study

15	Name	Sequence (N-->C)
	REEE	RGDYRYDY <u>T</u> FREEE-GLGSRYDYRGDY
	KEEE	RGDYRYDY <u>T</u> KEEE-GLGSRYDYRGDY
	SELE	RGDYRYDY <u>T</u> ASELE-GRGTRYDYRGDY
	TAQE	RGDYRYDY <u>A</u> PTAQE-AGEGPRYDYRGDY
20	ISQE	RGDYRYDY <u>P</u> TI <u>S</u> QE-LGQRPRYDYRGDY
	VSQE	RGDYRYDY <u>P</u> TVSQE-LGQRPRYDYRGDY

If desired, peptide scaffolds may also be formed with a predetermined shape or volume (Figs. 9A-9C). To form a scaffold with a desired geometry or dimension, an aqueous peptide solution is added to a pre-shaped casting mold, and

the peptides are induced to self-assemble into a scaffold by the addition of an electrolyte, as described herein. The resulting geometry and dimensions of the macroscopic peptide scaffold are governed by the concentration and amount of peptide solution that is applied, the concentration of electrolyte used to induce assembly of the scaffold, and the dimensions of the casting apparatus.

If desired, the peptide scaffolds formed from any of the above peptides may be characterized using various biophysical and optical instrumentation, such as circular dichroism (CD), dynamic light scattering, Fourier transform infrared (FTIR), atomic force microscopy (ATM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) (see, for example, Leon *et al.*, *supra*; Holmes *et al.* (2000), *supra*). For example, biophysical methods may be used to determine the degree of beta-sheet secondary structure in the peptide scaffold. Additionally, filament and pore size, fiber diameter, length, elasticity, and volume fraction may be determined using quantitative image analysis of scanning and transmission electron microscopy. The scaffolds may also be examined using several standard mechanical testing techniques to measure the extent of swelling, the effect of pH and electrolyte concentration on scaffold formation, the level of hydration under various conditions, and the tensile strength.

Peptide Scaffold Encapsulating Chondrocytes

A peptide with the amino acid sequence n-KLDLKLDLKLDL-c (KLD12) was synthesized using a peptide synthesizer (Applied Biosystems) and lyophilized to a powder. A 0.5% peptide casting solution was obtained by dissolving KLD12 in a solution of 295 mM sucrose and 1 mM HEPES. Freshly isolated

chondrocytes from bovine calf femoropatellar groove cartilage were re-suspended in the casting solution at a concentration of 15×10^6 cells/ml. The suspension was injected into a casting frame consisting of a 40 x 40 x 1.5 mm window supported on both faces by filter paper and a porous mesh. The casting frame was placed in

5 a 1 X phosphate-buffered saline (PBS, which contains 150 mM NaCl and 10 mM sodium phosphate at pH 7.4) bath for 15 minutes to induce the self-assembly of the peptides into a scaffold. Preferably, the cells are incubated in the sucrose solution for less than 5 minutes, or more preferably for less than 1 minute, before PBS is added. If desired, formation of a peptide scaffold may be confirmed using

10 phase-contrast microscopy. As a control, cells were also suspended into warm agarose (2% solution, w/w), injected into the casting frame, and placed into a cold 1 X PBS bath for 5 minutes. Both the peptide and control agarose gels were maintained in DMEM media (Gifco) plus 10 % FBS, which was changed every other day.

15 Initial cell viability was determined based on ethidium bromide staining using a standard FDA assay (Jones *et al.*, Journal of Histochemistry and Cytochemistry 33(1):77-79, 1985; Beletsky *et al.*, Journal of Immunological Methods 134(2):201-205, 1990). For both the peptide scaffold and the agarose gel, initial cell viability was comparable (80-95% after two hours and

20 approximately 75% after 24 hours).

For the following studies of protein and proteoglycan synthesis, glycosaminoglycan (GAG) accumulation, and immunohistochemistry, a 3 mm diameter by 1.5 mm thick cylindrical plug was punched immediately prior to addition of a radiolabel, digestion, or fixation. Extracellular protein production in

25 a plug from the scaffold was measured by addition of [³H]-proline to the media.

The radiolabeled proline was taken up by the cells and incorporated into newly synthesized proteins. After 16-24 hours in the radiolabeled media, the plug was rinsed with buffer to remove free [³H]-proline. The extracellular protein was digested by incubation in a proteinase K solution overnight at approximately

5 60 °C, and the radioactivity present in the digested protein was quantitated by scintillation counting. Proteoglycan production was measured similarly, except that [³⁵S]-sulfate was added to the media instead of [³H]-proline. The total accumulation of GAG, a proteoglycan component, was measured based on fluorometric analysis of the amount of DMMB dye bound (Chandrasekhar *et al.*,
10 Analytical Biochemistry 161(1): 103-108, 1987). The rates of protein and proteoglycan synthesis by the cells in the peptide scaffold were similar to the rates by cells in the agarose gel (Fig. 2). As total GAG accumulation increased (based on measurements of DMMB binding, Fig. 3), the rate of GAG synthesis decreased (based on radiolabel incorporation, Fig. 2), as seen previously.

15 For histological analysis of GAG, collagen I, and collagen II, samples were fixed at day 21. To visualize GAG, toluidine blue dye was applied using standard procedures (Fig. 4). Based on this staining, proteoglycan deposition is present throughout the gel, with higher intensity in the pericellular regions.

Immunohistochemical staining of collagen I using standard procedures resulted in
20 light background staining throughout the gel, with no increase in the pericellular region (Ioannidis *et al.*, Cell Tissue Res. 297:141-147, 1999; Domm *et al.*, Orthopäde 29:91-99, 2000). Collagen II staining with DMP showed a similar deposition pattern as that of GAG staining but with less defined pericellular staining (Ioannidis *et al.*, *supra*; Domm *et al.*, *supra*) (Fig 5). This result is
25 consistent with the known lower pericellular deposition of collagen.

For mechanical testing of the peptide scaffold, a 6 mm diameter by 1.5 mm thick cylindrical plug was taken from the scaffold at day 28. The plug was subjected to various levels of compression and the level of stress was measured, as described previously (Buschmann *et al.*, *supra*). (Figs. 6 and 7). Based on these results, the equilibrium modulus for the scaffold containing chondrocytes was 27 kPa compared to only approximately 0.5 kPa for a scaffold without cells or a scaffold immediately after encapsulation of chondrocytes. If desired, the stiffness of the peptide scaffolds may be further increased by subjecting the scaffold to static or dynamic compression using standard methods, such as those described by Buschmann *et al.* (*supra*). For example, dynamic compression at 0.01 to 3 Hz, superimposed on a static offset compression may be used. Typically, the dynamic strain amplitude is between 0.01 and 10%, and the static offset compression is between 5 and 15%.

The above method for encapsulating chondrocytes in a peptide scaffold was also repeated using a lower initial cell density of approximately 0.5×10^6 cells/mL. After formation of the peptide scaffold, the cells were substantially evenly dispersed in the scaffold, and cell viability was approximately 80% at 24 hours after scaffold formation. As illustrated in Fig. 8, pairs and clusters of cells that originated from a single cell were also substantially uniformly dispersed in the scaffold five days after its formation. In addition, the total number of cells increased approximately three-fold by day 5.

Other self-assembling peptides may be used in this method to encapsulate living chondrocytes or other cell types. If desired, the potential cytotoxicity of various peptide scaffolds may also be determined by measuring the rate of ^3H -thymidine incorporation due to DNA replication or the rate of RNA expression

of genes, such as actin or tubulin. Alternatively, a specific marker gene, such as enhanced green fluorescent protein under a specific promoter control, may be introduced into the cells to facilitate monitoring of gene expression and cell viability. The expression of proteins (*e.g.*, fibronectin and fibronectin receptors) 5 may also be analyzed using specific antibodies (see, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, Chapter 9, John Wiley & Sons, New York, 2000).

***In Vivo* Immune and Inflammatory Responses**

The *in vivo* immune and inflammatory responses to two self-assembling peptides were analyzed. Neither the RAD16 or EAK16 peptides, alone or conjugated with other highly immunogenic proteins such as BSA, elicited a detectable immunological response when injected into rabbits or goats (Holmes *et al.* (2000), *supra*). Also no significant titers of antibodies were obtained. To measure the inflammatory response elicited by these peptides, the peptides were injected into the leg muscle and brain of rats (Holmes *et al.* (2000), *supra*). No inflammation in these or neighboring areas was observed during the two weeks following the injection. Other self-assembling peptides may be tested similarly to measure the immune and inflammatory responses that they generate.

The lack of an immune or inflammatory response to these peptides in a variety of mammals suggests that the peptides may not elicit an adverse immune or inflammatory response when administered to humans. Furthermore, structural modeling and theoretical analysis of peptide presentations by class I and class II MHC molecules also suggest that the self-assembling peptides of the present invention are not likely to elicit strong immune response due to their alternating

distribution of charged and uncharged residues.

In Vivo Animal Model Studies.

Several previous studies have employed a canine model to compare the reparative tissues formed in defects in articular cartilage (see, for example,

5 Brittberg *et al.*, New England J. of Med. 331:889-894, 1994; Breinan *et al.*, J. Bone Jt. Surg. 79-A: 1439-1451, 1997; Breinan *et al.* Tiss. Engr. 4:101-114, 1998; Nehrer *et al.*, Biomaterials 19:2313-23128, 1998). For initial studies, four dogs are tested for each peptide scaffold. Two defects are made in each knee, and each defect is filled with a peptide scaffold encapsulating chondrocytes. Because 10 previous studies have generated data on untreated control groups, an untreated control group is not needed for this study (Breinan *et al.* (1997), *supra*). However, if desired, dogs in which one or more of the knee defects are not filled with a scaffold or are filled with a scaffold that does not contain cells may be used as controls.

15 The power calculation for determining the required sample size for these experimental groups is based on detecting a 30% difference in the mean values of total fill, the areal percentage of hyaline cartilage, and the values of specific mechanical properties, assuming a 25% standard deviation. A 30% change in the outcome variable is expected to be a meaningful indication of the benefit of one 20 treatment group over another. For a power of 0.80 ($\beta=0.20$) and a level of significance of $\alpha=0.05$, $n = 8$ specimens are required. The statistical analysis is performed by averaging the values for the two defects in the same knee and counting the average value as one observation. However, in our previous work with this animal model, there were no systematic relationships between the two

defects in the same knee of an animal. Thus, if desired, the individual defects may be treated as independent observations.

For this study, adult mongrel or hound dogs, each weighing approximately 25-30 kilograms, are used. Prior to the operation, the knee joints are examined

5 roentgenographically to exclude animals with degenerative joint disease. All operations are performed under general anesthesia and sterile conditions, as described previously (Breinan *et al.* (1997), *supra*). Two 4 mm diameter defects are created in the trochlear groove of the right stifle (knee) joint. These defects are placed approximately 1.25 and 2.25 centimeters proximal to the intercondylar
10 notch, each slightly lateral or medial to the midline. A 4 mm diameter dermal punch is used to outline the defect. Using loupe visualization, an attempt is made to remove all non-calcified cartilage from the defect by scraping the calcified cartilage surface with a customized curette, without fissuring the calcified cartilage. The objective is to remove all of the articular cartilage and to gently
15 scrape the calcified cartilage to facilitate the integration of the reparative tissue with the calcified cartilage. A peptide scaffold with or without encapsulated chondrocytes is placed in each defect. Before closing the capsule, bleeding vessels are clamped and cauterized. The knee joint is closed by zero point suturing. Postoperatively, the operative knee are immobilized by external fixation
20 (IMEX Veterinary, Longview, Texas) for ten days (Breinan *et. al.* (1997), *supra*). It is also contemplated that the operative knee may be mobilized for a longer time period if required for the peptide scaffold to increase in stiffness as extracellular matrix components are secreted *in vivo* by the encapsulated cells and/or nearby cells. Six months after the first surgical procedure, two defects are made in the
25 left knee using the same procedure. The dogs are sacrificed 12 months after the initial surgical procedure, producing a postoperative evaluation period of 12

months for the right knees and six months for the left knees. It is also contemplated that other postoperative periods may be used, such as a few hours, a few days, or even a few years. Additionally, the defects in other dogs may be analyzed at earlier time points, such as after 30 minutes, a few hours, or a few

5 days, to determine if early displacement of the graft is occurring.

After formalin fixation, specimens are immersed in a 15% disodium ethylenediamine tetracetate decalcifying solution at pH 7.4. The samples are placed on a shaker at 4 °C for four weeks, and during this incubation the decalcifying solution is changed every week. Samples are rinsed thoroughly,

10 dehydrated, and embedded in paraffin at 60 °C. Seven-micrometer thick sections are stained with hematoxylin, eosin, and/or safranin O/fast green. Selected paraffin sections are stained with antibodies to type I collagen and type II collagen.

The specific tissue types filling the defects are determined by evaluating the percentage of the area of the central section through the defect occupied by each tissue type: articular cartilage, non-articular hyaline cartilage, fibrocartilage, and fibrous tissue (Breinan *et al.* (1997), *supra*). These percentages refer only to the representative histological cross-section through the middle portion of the defect. They do not imply values equivalent to the actual volume percentages of tissues in

20 the defects. Due to edge effects (regenerating tissues tend to form at the periphery of the defect), only sections representing 60% or more of the defect diameter are analyzed. Sections taken too close to the edge of the defect may preferentially show regeneration, which could yield misleading data. The effects of treatment and time on the areal percentages of specific tissue types are determined by
25 two-way ANOVA. Group comparisons are made using the Student t test with the appropriate corrections.

Additionally, any degradation of adjacent tissues and the bonding of the repaired tissue to the subchondral plate and the adjacent cartilage may be evaluated. The presence of new tissue formed in the remodeling subchondral bone underlying the defects may also be determined, and the area surrounding the

5 defects may be analyzed for signs of inflammation. If desired, the rate of scaffold degradation may be measured. For this determination of the *in vivo* degradation rate, radiolabelled peptides, such as ¹⁴C-, ³H-, or ³⁵S-labelled peptides, may be assembled into a radiolabelled peptide scaffold and administered to a mammal using the methods of the present invention. At one or more time points after

10 administration of the radioactive scaffold, urine or blood samples are obtained from the mammal. The amount of radioactivity in the sample is measured to determine the amount of degradation products that have been released from the scaffold.

Other animal models may be used to test peptides scaffolds encapsulating living cells for the ability to repair or replace tissues *in vivo*. For example, scaffold encapsulating chondrocytes may also be tested using rabbit models of cartilage defects (see, for example, Perka *et al.* Clinical Orthopaedics 378:245-254, 2000; Solchaga *et al.*, Journal of Orthopaedic Research 18(5):773-780, 2000). Standard bone tissue engineering animal models may be used for *in vivo* studies of scaffolds encapsulating cells such as osteocytes (see, for example, Lennon *et al.*, Experimental Cell Research 219(1):211-222, 1995; Solchaga *et al.*, *supra*; Boyan *et al.*, Journal of Orthopaedic Research 17(2):246-55, 1999). Examples of ligament tissue engineering animal models that may be used to test peptide scaffolds for the ability to repair or replace ligament tissue *in vivo* include those described by Awad *et al.* (Tissue Engineering 5(3):267-277, 1999) and Kato *et al.* (Journal of Bone and Joint Surgery (Am)

73(4):561-574, 1991). Peptides scaffolds encapsulating any other cell type may also be routinely tested in an appropriate animal model. Standard medical procedures may be used to adapt the methods used to repair or replace tissues in these animal models for the treatment of other mammals, such as humans.

5

Other Embodiments

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually indicated to be incorporated by reference.

10 While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations following, in general, the principles of the invention and including such departures from the present disclosure within known or customary practice 15 within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is: